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Occurrence of Citrus Greening disease in Acid Lime [*Citrus aurantifolia* (Christm) Swingle] orchards in Pune (Maharashtra), India

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A B S T R A C T

Citrus is an important fruit crop in India. Citrus greening disease caused by a fastidious bacterium (*Candidates Liberibacter asiaticum*) is one of the important diseases that limit the citrus fruit production in several part of the country including Maharashtra. The greening disease is prevalent in sweet orange in western part of Maharashtra and in Nagpur mandarin in Central and North Western part of the Maharashtra. A survey was conducted during January 2008 in Pune district of Maharashtra. Wild mottling symptoms were observed in acid lime orchard which indicates that the incidence as greening was more on sweet orange as compared to acid lime. The presence of greening bacterium was confirmed by Polymerase chain reaction and nucleotide sequencing. Sequence analysis indicated that the greening bacterium is *Candidates Liberibacter asiaticum*). This is the first confirmed report of presence of greening bacterium in acid lime.

Introduction

Citrus is native to a large area, which extends from India to China, Philippines, Burma, Thailand, Indonesia, Nigeria, Colombia, Guinea, Saudi Arabia, New Caledonia. There are some popular species of citrus group known as Kagzi lime (*Citrus aurantifolia*), Grapefruit (*Citrus paradisi*) and Pummelo (*Citrus grandis*), Sweet orange (*Citrus sinensis*), Mandarin (*Citrus reticulata*). In India, citrus is the third largest fruit industry after Banana and Mango. In India, Citrus fruits are successfully grown in Andhra Pradesh, Gujarat, Maharashtra, Karnataka, Uttarakhand, Bihar, Assam, Rajasthan, Madhya Pradesh and other states. They are the most common fruits in India and have become popular because of their availability almost throughout the year at moderate prices.

Among the citrus group, Kagzi lime is one of the most important horticultural crops in India. Kagzi lime (Pati lime, Spur lime, Acid lime, Mexican lime) in Hindi is known as 'Neebu or Nimbu', whereas in Gujarati, it is known as ‘limbu’, which belongs to family Rutaceae. Lime (*Citrus aurantifolia*) is the

most important fruit crop in India as the demand for its consumption is very high due to the nutritional value and its availability at cheap prices. Lime is popular for its beautiful appearance and pleasing flavor and for its excellent food qualities. Lime does not edible in same manner as the other Citrus species.

Citrus greening disease (CGD) is an important disease of citrus and affects the production of citrus fruits in several parts of India. The disease has been reported from several Asian and African countries (Garnier and Bove, 1996). The disease has been reported in different citrus species in different parts of India presence of citrus greening bacterium disease. Varma *et. al*., (1993) used NA hybridization to confirm the greening bacterium disease in A.P. Delhi, Maharashtra, Karnataka and Orissa Baranawal *et al.,* 2005 detected citrus greening bacterium in Sweet orange (Delhi) mandarin orange, (Maharashtra) and grapefruit (Jammu. Among the commercial cultivars, group of sweet oranges [(*Citrus sinensis* (L.) Osbeck] is more sensitive to the greening bacterium than the groups of lemons [*C. limon* (L.) Burm.f.] and limes [(*C. aurantifolia* (Christm.) Swingle] (Ahlawat, 1997). The symptoms are often confused with nutritional deficiencies and other stress related factors. It is transmitted by an Asian citrus psylla (*Diaphorina citri)* in India (Capoor *et al.,* 1967). In the present study by PCR presence of citrus greening bacterium was confirmed in acid lime orchard grown in western Maharashtra.

Materials and Methods

Collection of samples

Survey of acid lime and sweet orange orchards was done during January, 2008 at Pune district of Maharashtra. Symptomatic leaf samples were collected from citrus plants of Acid lime (*Citrus aurantifolia)* and sweet orange (*Citrus reticulate*)

DNA extraction

Total DNA was extracted using Sodium sulphide methods (Baranawal *et al.,* 2003) from 100 mg midrib of citrus leaves showing yellowing / yellow mottling symptom as well as from healthy citrus leaves.

Polymerase Chain Reaction (PCR) for citrus greening bacterium

5 µL of extracted DNAt was used in PCR for detection of Cla. PCR was performed in a 50 µL reaction mix containing 0.2 µM each of forward and reverse primer of Cla (5'TGGGTGGTTTACCATTCAGTG, 5'CGCGACTTCGCAACCCATTG), *Taq* DNA polymerase 5 U (Promega, Madison, USA), 5 µL of 10 x PCR buffer, 1 µL of 10mM dNTPs, and 3µL of $MgCl_2$ 25 Mm. Samples were amplified for 30 cycles, using a Mastercycler (Eppendorf, Germany). Each cycle consisted of denaturation at 94 ^oC (30s), primer annealing at 58 ^oC (60 s), extension at 72 ^oC (60s), with a final extension of 10 min at 72 ^oC. 10 µL of amplified product were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide at a concentration of 0.5 $\mu g \cdot mL^{-1}$ and photographed under UV illumination with an imaging system (Biorad XR documentation system). All the experiments were repeated at least twice.

Sequencing

The PCR product from acid lime was Purification from the QlAquick PCR Purification kit (Qiagen Gmbh, Hilden, Germany) Direct purified sequence from Bangalore Geni Bangalore. BLAST programmed was used to compare the homology with sequences available in the GenBank database [M94319 (*Liberibacter* asiaticus, Pune India), U09675 (*L*. africanus nelspruit, S. Africa) and AF2484 (*L*. africanus

subsp *capensis*, S. Africa) Sequences were aligned using CLUSTAL W and the sequence identity matrix for pair wise combination of aligned sequences was calculated with Bio Edit Sequence Alignment Editor (Hall, 1999).

Results and Discussion

Results are presented in table1, 2 and Figure 1 and 2. Incidence of greening disease on acid lime was observed up to 10% of citrus planted in 5 orchards. Figure 1 shows that approximately 451 –bp (lane 1, 2) amplicon was obtained from 2 samples out of 3 citrus plants showing symptoms of yellowing and mottling. Positive samples were from sweet orange (Lane 4) Healthy plant of Sweet orange was used as negative control and did not produce any amplified product (lane 5). Figure 2 shows approximately 451 –bp (Lane 2, 3, 4, 5) amplicon was obtained. Lane 6 Positive Control and plant Lane 7 Healthy plant of Sweet orange.

Table.1 Occurrence and distribution of greening disease in Acid lime and Sweet orange orchard

Sr. No	Name of Nursery	Acid lime samples	PCR results	Sweets orange samples	PCR Results
1	IARI, Regional station, Pune	5	1/5	5	1/5
2	Sairam Nursery, Baramati	5	0/5	5	0/5
3	Kanhari Nursery Baramati	5	1/5	5	1/5
4	Govt Nursery Baramati	5	0/5	5	0/5
5	Yaswant Nuesery Baramat	5	1/5	5	1/5
6	Sadanand Nursery Baramati	5	1/5	5	1/5

Table.2 Validation of Citrus greening disease in Acid Lime and sweet orange orchard

Place	**Time of sample collected**	**Name of Nursery**	**Name of citrus Species**	**Number of sample collected**	**PCR result**
Pune	January 2008	IARI, Regional station, Pune	Acid lime	10	+2
Pune	January 2008	IARI, Regional station, Pune	Sweet orange	10	+3

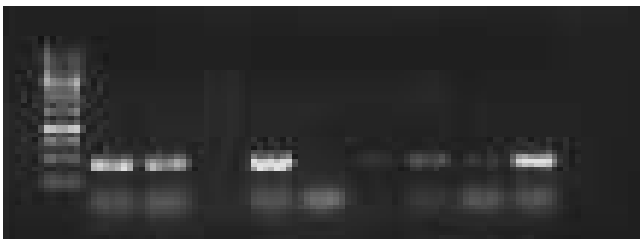

Fig.1 PCR results of Citrus greening disease in acid lime. Lane M Marker one kb ladder: Lane 1 & 2 acid lime (Pune): Lane 3, 4 Sairam nursery, Lane 5 Kanhari nursery, Baramati: Lane 6 Govt nursery, Baramati: Lane7, 8 Yashvant nursery Baramati: Lane 9 Sadanand nursery Baramati: Lane 10 Healthy sample.

Fig.2. PCR results of Citrus greening disease in acid lime and Sweet orange. Lane M Marker one kb ladder: Lane 1 to 2 (acid lime); Lane 3 to 5(Sweet orange) Lane 6 Positive control lane 7.Healthy Control.

The size of the PCR products are similar to that amplified from *Candidatus Liberibacter asiaticus* as also shown by Baranwal *et al.*, 2005. This was further confirmed when the sequence analysis showed that the cloned DNA from both the samples of mandarin orange from Delhi India were found to be 451 long (GenBank accession no. AY266352) having 100% sequence identity with the corresponding nucleotide sequence of ribosomal protein gene *Candidatus Liberibacter asiaticus* (GenBank accession no. M94319). The sequence analysis showed that ribosomal protein gene sequence from *Candidatus Liberibacter asiaticus* from India (Poona and Delhi) and Bhutan were completely identical with each other and showed 89% and 79% identity with *L. africanus* nelspruit and L. *africanus subsp capensis* respectively. This study demonstrated that the primers from 16s rDNA can be used for specific detection of *Candidatus Liberibacter asiaticus*, the agent for citrus greening disease and should be highly useful in phytosanitary assay and bud wood certification.

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